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Quantification of fipronil and its metabolite fipronil sulfone in rat plasma over a wide range of concentrations by LC/UV/MS

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ABSTRACT

Fipronil is an insecticide extensively used to treat pets, which has been identified as a potential thyroid disruptor in the rat. In this species, fipronil is mainly metabolized to fipronil sulfone and plasma concentrations of fipronil sulfone can be at least 20-fold higher than those of fipronil. Investigations of fipronil and fipronil sulfone exposure in blood remain sparse because of the lack of convenient and suitable analytical methods. We have developed and validated an LC/UV/MS/MS method to quantify both fipronil and fipronil sulfone within a wide range of concentrations in rat plasma. The double detection UV and MS coupled on-line enabled the concentrations to be measured over a 3 Log range (2.5–2500 ng/mL). The volume of sample required for the extraction by solid phase extraction was reduced to 75 μL with a recovery higher than 70%. The two-detection method agreement, evaluated with a Bland–Altman plot, was good for concentrations between 50 and 150 ng/mL. The method was applied to monitor plasma concentrations following a commonly used dosage regimen for the toxicological evaluation of fipronil in rats.

1. Introduction

Fipronil is a phenylpyrazole insecticide used in phytosanitary and veterinary medicinal products [1]. It is mainly metabolized to fipronil sulfone in rat and human [2]. Since 2004, its utilization as a phytosanitary product has been suspended in France because of its possible implication in honeybee mortality [3]. However, it remains one of the most widely used ectoparasiticides for pets.

To date, there are few evaluations of fipronil toxicity [2]. It has been reported that fipronil treatment can be associated with thyroid disruption in the rat [4]. However, in all these toxicological evaluations, there is a lack of information concerning the internal exposure (i.e. blood concentrations) of the animal to fipronil and/or its metabolite [3]. It is therefore very difficult to link the adverse effect on thyroid function to specific levels of blood concentration and to assess the relevance of these observations to the level of internal concentration observed in the human population. Furthermore, results obtained from cultured human and rat hepatocytes suggest that important quantitative differences might occur between rat and human in terms of fipronil metabolism [2]. This could be a major source of difference in the pattern of exposure between the two species and might impact on the extrapolation of rat results to man. Thus, characterizing rat exposure is critical for the assessment of the relevance of rat results in evaluating the risk of fipronil for human health. The only available data concerning fipronil exposure in rat relies on pharmacokinetic investigations using radiolabeled fipronil that did not enable fipronil to be discriminated from its metabolites [3]. One limiting factor for the characterization of exposure in toxicological evaluation might be the lack of available assay methods for plasma that fulfill the requirements of bioanalytical methods in the pharmaceutical industry [5]. Indeed, fipronil has been mainly quantified by gas or liquid chromatography coupled to mass spectrometry [6–8] or electron capture detection [9] in honey, pollen, water or soil samples [8,9]. A GC–MS/MS method, validated according to the European standard 2002/657/EC, has been described for screening residues in ovine plasma [6]. However, the criteria to validate a method according to this standard differ from the validation requirements of a bioanalytical method dedicated to pharmacokinetic (PK) studies. We previously developed an HPLC method with UV detection [10] for fipronil quantification in rat plasma and showed that fipronil was actively metabolized to its sulfone derivative. As a consequence, after a repeated oral administration, fipronil concentrations in rat plasma at steady state were lower than the assay limit of quantification (<100 ng/mL) whereas fipronil sulfone concentrations ranged from 1 to 2 μg/mL. Moreover, for these methods, sample preparation necessitated large volumes (>100 μL) of plasma. The volume of the plasma samples obtained is often limited in rodents in PK studies requiring serial samples.

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Table 1
UV and SRM parameters used for fipronil and sulfone analysis.

	UV detection		MS detection		
	$tR \pm$ SD (min)	λ_{abs} (nm)	$tR \pm$ SD (min)	MS transition	Collision energy %
Ethiprole	7.80 ± 0.03	280	8.07 ± 0.05	395 < 359	15
Fipronil	10.65 ± 0.06	280	10.95 ± 0.06	435 < 399	22
Sulfone	12.23 ± 0.11	280	12.52 ± 0.09	451 < 415	25

Consequently, we decided to develop and validate, according to the FDA guidelines, an original HPLC method coupled on-line to UV and MS detection, allowing the quantification in the same run of a wide range of concentrations of fipronil and its main metabolite fipronil sulfone in rat plasma.

2. Materials and methods

2.1. Chemicals

Fipronil (CAS-No. 120068-37-3) and fipronil sulfone (CAS-No. 120068-36-2) were purchased from Accustandard®, Inc. (New Haven, USA). Ethiprole, formic acid, acetic acid, methanol (LC–MS grade) and acetonitrile were from Sigma–Aldrich (Saint Quentin Fallavier, France). Water was obtained from an ultrapure water (18.2 MΩ) system (Elga Labwater Veolia, Anthony, France).

Working concentrated solutions were prepared by serial dilutions of fipronil and fipronil sulfone reference standards (100 μg/mL) in MeOH/H_2O (50/50, v/v) in order to obtain final concentrations of a mixture fipronil/fipronil sulfone at 10, 1 and 0.1 μg/mL.

Standard and quality control (QC) solutions were obtained by diluting working solutions in drug-free rat plasma to obtain concentrations of 2.5, 5, 10, 25, 50, 100, 250, 500, 1000 and 2500 ng/mL for the standard curve calibrators and 15, 40, 150, 800 and 1800 ng/mL for QC samples. Calibrators and QC were stored at −20 °C in 100 μL aliquots.

The stock solution of the internal standard (IS), ethiprole, was prepared in acetonitrile at a concentration of 1 mg/mL and stored at 4 °C. A new working IS solution (10 μg/mL) was prepared each week by diluting the stock solution in ultrapure water.

2.2. Extraction procedure

Fipronil and fipronil sulfone were extracted from rat plasma using C8 solid phase extraction (SPE) cartridges (Bond Elut® C8, 100 mg, Varian, Palo Alto, CA, USA) under vacuum. Plasma samples (75 μL) spiked with 75 μL of IS were applied to the cartridges conditioned with 1 mL of MeOH and 1 mL of ultrapure water. The cartridges were washed with 1 mL of H_2O/AcN (95/5) and the analytes were eluted with 1 mL of methanol acidified with 1% acetic acid. The extract was dried at 40 °C under nitrogen and reconstituted in 75 μL of MeOH/H_2O (50:50, v/v). The resulting extract solution was vortex mixed and centrifuged at 20,000 × *g* for 10 min at 4 °C. The supernatant (50 μL) was collected in HPLC vials and 10 μL was injected into the system.

2.3. Instrumentation and analytical conditions

Chromatographic analyses were performed on a Thermofinnigan Surveyor® HPLC system with diode array detector (DAD) and a LCQ Deca XP Max® ion trap mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA). Separation was carried out with a Hypersil-BDS® C18 column (150 mm × 2.1 mm; 3 μm, Thermo) and a C18 guard column (10 mm × 2.1 mm, Thermo) at 40 °C. A gradient elution was used at a flow rate of 150 μL/min with a mobile phase of MeOH, 0.05% formic acid (A) and H_2O, 0.05% formic acid (B) at the following conditions: 0 min, 60% A; 0–3 min, 70% A; 3–14 min, 70% A; 14–14.5 min, 60% A; and 14.5–22 min, 60% A.

The DAD wavelength was set at 280 nm and molecules were ionized with an electrospray ionization source in negative mode (ESI^-). The spray needle was set at a potential of 5 kV. Capillary voltage and temperature were −13 V and 300 °C, respectively. Sheath gas and auxiliary gas flow rate of nitrogen were set at 44 and 10 (arbitrary units), respectively. The tube lens offset was set at −35 V and helium was used in the trap as damping and collision gas.

Fipronil, fipronil sulfone and ethiprole were detected by selected reaction monitoring (SRM) mode with the parameters reported in Table 1. Chromatographic data were monitored by Xcalibur® 1.4 software (Thermo Electron Corporation).

Statistical analyses were performed using WinNonlin® 5.2 software (Pharsight Corporation, Mountain View, CA, USA).

2.4. Characterization of rat internal exposure

A dose of 3 mg/kg body weight (b.w.) of a fipronil suspension in methylcellulose was administered daily by intragastric gavage for 13 days to three female Wistar rats (280 ± 13 g). Blood samples (200 μL) were collected in lithium heparinated tubes before and at 1, 4, 6, 10 h after the first and the last administrations using a catheter inserted under anaesthesia in the left femoral vein. The samples were centrifuged for 30 min at 4 °C and 5000 × *g* within 2 h following sampling and 100 μL aliquots of plasma were stored at −20 °C.

Animal procedures were conducted in accordance with accepted standards of humane animal care under agreement number 31-242 for animal experimentation from the French Ministry of Agriculture.

3. Results and discussion

3.1. Optimization of the assays

3.1.1. Choice of the internal standard

In a bioanalytical method, the internal standard (IS) should be structurally related to the analytes of interest to ensure similar behaviour of the compound during extraction and assay procedures. Ideally, in mass spectrometry, the internal standard is a stable isotope-labeled of the analyte to obtain an identical ionization pattern as the analyte at the same retention time. In mass spectrometry, despite their co-elution, the analyte and its isotope will be differentiated by their respective masses. While with UV detection, they could not be separated on the chromatogram, so this kind of internal standard was not suitable for this method. In previous studies [6], phenylpyrazole compounds were tested as potential internal standards, in particular the 5-amino-1-phenylpyrazole-4-carbonitrile (Fig. 1) which has a similar behaviour to fipronil in the extraction procedure. However, the authors reported major variations at the highest concentration levels of fipronil and fipronil sulfone because the internal standard was not ionized in the same way as the analytes in mass spectrometry. We focused our choice on another phenylpyrazole insecticide: ethiprole which differs from fipronil by one $-C_2H_5$ group instead of one $-CF_3$ group on the sulphinyl function (Fig. 1) [11]. To date,

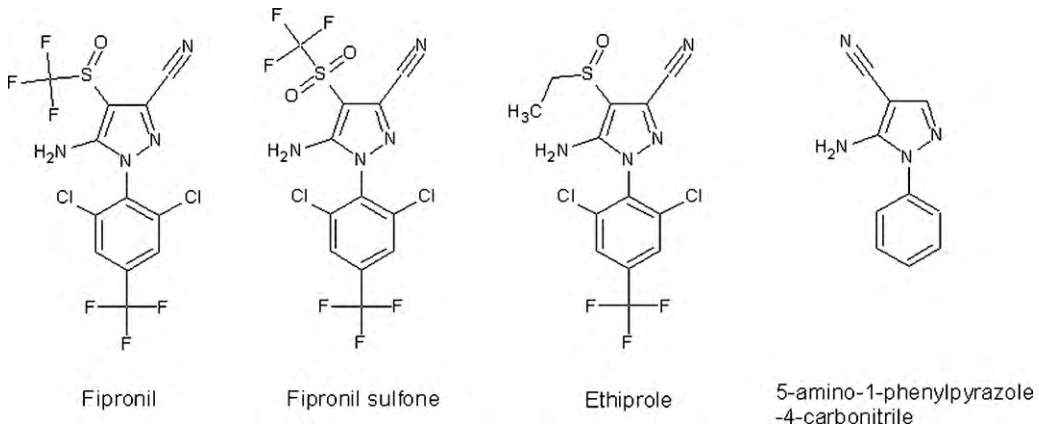

Fig. 1. Chemical structure of fipronil, fipronil sulfone, 5-amino-1-phenylpyrazole-4-carbonitrile and ethiprole (internal standard).

ethiprole has not been described as a fipronil or fipronil sulfone metabolite in rat plasma and urine [12].

3.1.2. Optimization of the LC/UV/MS conditions

Fipronil, fipronil sulfone, and the internal standard ethiprole were separated on a C18 column with an acidic gradient elution. In MS detection, it is generally assumed that acidic conditions decrease ionization in the electrospray negative mode. However, for fipronil and fipronil sulfone, ionization was not influenced by the addition of 0.05% formic acid in the mobile phase and gave a more reproducible peak response than without the addition of formic acid. This better reproducibility might be explained by the fact that under acidic conditions, HPLC separation was improved and thus fewer components co-eluted with the analyte into the ESI source leading to an elimination of potential cross suppression/enhancement effects [13].

As samples were first run through UV detection, we evaluated the potential degradation of fipronil and sulfone submitted to UV emissions. A standard solution of the mixture at 100 ng/mL was analyzed in triplicate, in LC/UV/MS and in LC/MS alone. At 100 ng/mL, there was no significant loss of signal in MS detection after UV detection; the relative standard deviation (RSD%) between the two detections were lower than 7% and 5% for fipronil and fipronil sulfone, respectively.

One of the main issues when developing this double detection method has been to identify an IS concentration allowing a middle range response for both detection methods to be obtained. As UV detection is much less sensitive than MS, we first determined the IS concentration for this method and then the MS parameters were optimized to obtain a middle range MS response for this concentration. Collision energies were tested from 5% to 20% (arbitrary units). The best compromise between the two detection methods was obtained with a level of collision energy of 15%.

3.1.3. Optimization of the extraction procedure and matrix effect

One of our main goals was to reduce the plasma volume required for the assay while ensuring at the same time a limit of quantification within the ng/mL range. Fipronil and fipronil sulfone were extracted from rat plasma by solid phase extraction (SPE) with 75 μL volume samples. The matrix effect characterized by the matrix factor (MF), i.e. the ratio between the peak response of the analyte extracted from the matrix to the response of the analyte extracted from aqueous solution (in the absence of matrix ions) [14], was evaluated at different concentrations ranging from 2.5 to 1000 ng/mL. The matrix factor was close to 1 for all concentrations.

Moreover, the recovery, i.e. the ratio between the responses of the extracted samples to the responses of the standard in solution (without extraction), was evaluated at the LOQ and 10, 100 and 1000 ng/mL. The recoveries for fipronil and its sulfone metabolite were 90% and 77%, respectively with a RSD lower than 10%. Our extraction procedure optimized for a 75 μL sample volume fulfilled the criteria for bioanalytical method validation with recoveries higher than 70% and good repeatability (RSD < 15%) [5].

3.2. Validation of the methods

As the quantitative determination of drugs and their metabolites in biological fluids is directly related to the evaluation and interpretation of PK data, a reliable and reproducible determination of plasma concentrations is essential. The method was fully validated according to bioanalytical method recommendations described in the Food and Drug Administration (FDA) guidelines in terms of selectivity, linearity, repeatability, reproducibility and stability for both detection systems [5,14].

3.2.1. Selectivity

Selectivity of the methods was assessed by comparing the SRM chromatogram of a set of six blank samples with that of six samples spiked at the LOQ level (2.5 ng/mL for MS detection). As shown in Fig. 2, no peak was detectable at the retention times of fipronil and fipronil sulfone.

3.2.2. Linearity

Calibration curves were fitted using seven concentration levels ranging from 2.5 to 250 ng/mL with MS detection and over six concentrations ranging from 50 to 2500 ng/mL for UV detection. Each point of the two calibration curves was injected in triplicate within one day and peak area ratios of analyte area to IS area versus analyte concentrations were plotted using a linear regression. Linear ($Y = aX + b$) and quadratic ($Y = aX^2 + bX + c$) models were tested with weightings: 1, $1/X$ and $1/X^2$ (X = nominal concentration) [15]. The linearity of the calibration curve was assessed using three approaches [16]: (1) the calculation of the relative concentration residuals between nominal concentration and concentration estimated by the model (RCR%), which should be lower than ±15% (except at the LOQ ± 20%); (2) the visual inspection of the residual distribution which should be randomized around the mean; and (3) a lack of fit test to check the goodness of the fitting of the model.

For UV detection, the simple linear model weighted by $1/X^2$ fulfilled the three criteria of the three aforementioned approaches for both fipronil and its metabolite (Table 2). For MS detection and for both molecules, the RCRs were lower than ±15% and the lack of fit test was not significant ($p > 0.05$). However, the distribution of the

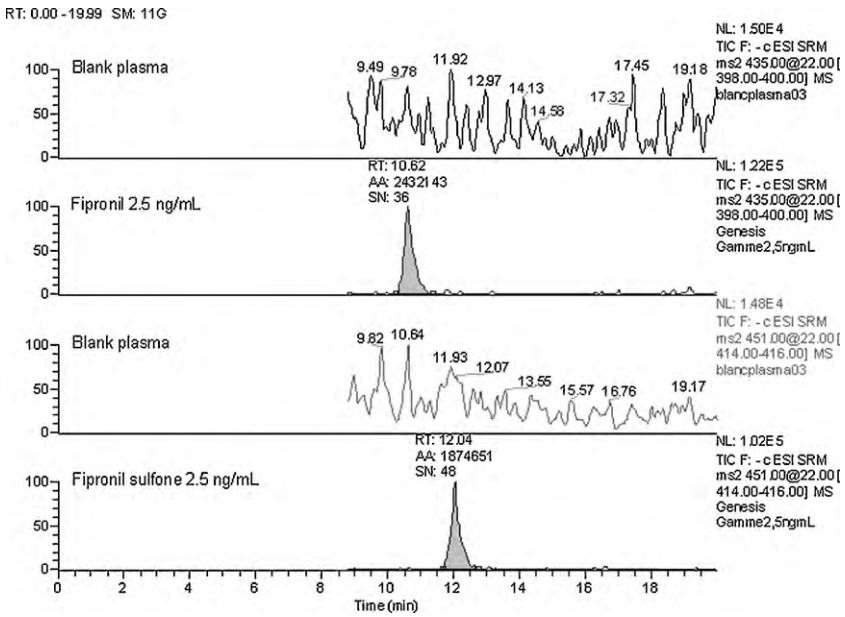

Fig. 2. SRM chromatograms of blank and spiked rat plasma (2.5 ng/mL of fipronil and 2.5 ng/mL of fipronil sulfone).

residuals for both molecules presented a specific "U" pattern, thus a quadratic model weighted by $1/X^2$ was finally selected.

3.2.3. Limit of quantification

The LOQ was established on six independent samples of standard with the calibration curve model determined previously. The LOQ were 2.5 and 50 ng/mL with MS and UV detection, respectively. The precision and accuracy of each LOQ are shown in Table 2. Our procedure gave sensitivity suitable for PK studies with MS detection. Furthermore, with UV detection, we improved the performance of the assay for the LOQ as compared with the previous study while reducing the required volume of sample [10].

3.2.4. Precision and accuracy

Intra-day and inter-day precisions and accuracy of fipronil and fipronil sulfone were calculated from three days and with six replicates of quality control (QC) samples at three different concentration levels (low, middle and high) covering the range of standard curve concentrations for each detection method. The results are given in Table 2. Precision was systematically lower than 15% and accuracy ranged from 90% to 104%. Thus, both parameters fulfilled the acceptance criteria of the FDA guidelines [5].

3.2.5. Stability

Long-term stability of fipronil and fipronil sulfone in plasma samples was checked by comparing the concentrations measured in QC samples stored for over one year at −20 °C with those of QC with short-term storage at −20 °C (less than one week). The difference in the mean concentrations measured for the two sets of sample accounted for 3% of the measured concentration of the short storage set.

Post-preparative stability, i.e. the stability during the residence time in the autosampler, was evaluated by assaying QC samples at 6, 12 and 24 h after extraction. The mean deviation between the concentration measured at the first injection and the concentration measured 24 h later was lower than 9% and 18% for fipronil and fipronil sulfone, respectively with mass spectrometry and lower than 5% and 8% with UV detection. Thus, the processed extracts were stable in the autosampler (at room temperature) for at least 24 h.

3.3. Agreement between UV and MS detection

The agreement between UV and MS detections coupled online was evaluated with the 150 ng/mL QC ($n = 24$), and the 50 ($n = 14$), 100 and 250 ng/mL ($n = 8$) calibration points used for the

Table 2
Validation results of fipronil and fipronil sulfone with UV and MS detection in rat plasma.

Detection	Nominal concentration (ng/mL)	Fipronil				Fipronil sulfone			
		Mean	Accuracy	Precision RSD%		Mean	Accuracy	Precision RSD%	
				Intra-day	Inter-day			Intra-day	Inter-day
MS	LOQ ($n = 6$)								
	2.5	2.7	108%	9%		2.54	102%	6%	
	QC ($n = 18$)								
	15	15.62	104%	6%	10%	13.8	94%	11%	13%
	40	40.66	102%	11%	15%	38.67	97%	10%	14%
	150	162.42	108%	9%	9%	153.14	102%	4%	8%
UV	LOQ ($n = 6$)								
	50	46.74	93%	10%		44.82	90%	5%	
	QC ($n = 18$)								
	150	148.68	99%	6%	8%	147.65	98%	6%	6%
	800	813.85	102%	5%	6%	795.21	99%	7%	7%
	1800	1880.52	104%	6%	6%	1878.38	104%	6%	7%

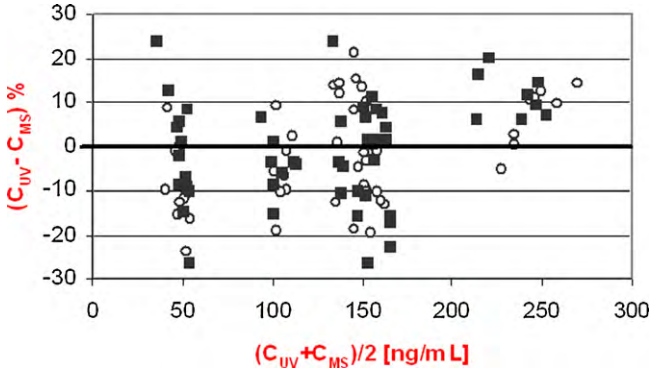

Fig. 3. Bland–Altman plots of fipronil (■) and fipronil sulfone (○) between UV and MS detection for the common concentrations (50, 100, 150 and 250 ng/mL). The *X*-axis is the mean of the concentrations measured with UV and MS detection and the *Y*-axis is the percentage difference between the two methods.

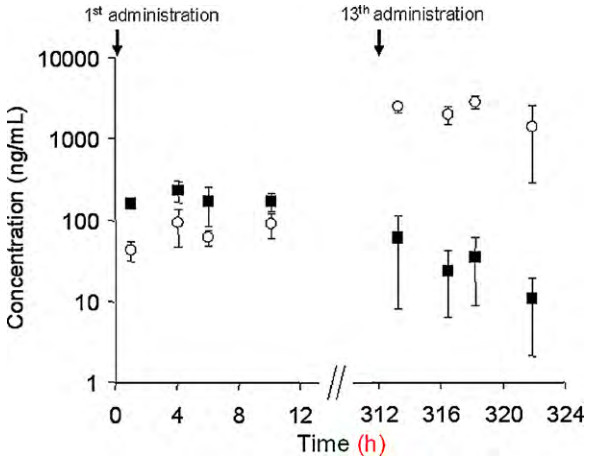

Fig. 4. Mean plasma concentration of fipronil (■) and fipronil sulfone (○) versus time after daily oral administration to three female rats, at the dose of 3 mg/kg b.w. fipronil and fipronil sulfone concentrations were assayed at 1, 4, 6, 10 h after the first and the last administrations.

validation. The Bland–Altman plots [17] for fipronil and fipronil sulfone enabled the two methods to be compared and to determine the threshold concentration for the choice of one or the other method. As shown in Fig. 3, the two methods appeared to be in good agreement for concentrations between 50 and 150 ng/mL with a randomized distribution around the 0% deviation. For concentrations higher than 150 ng/mL, the deviations between UV and MS detection were systematically positive suggesting that the quadratic model used for MS detection might underestimate the concentrations (% UV–MS > 0). Thus, the threshold concentration was set at 100 ng/mL.

3.4. Applicability of the method for pharmacokinetic studies

This method was developed for the measurement of fipronil and fipronil sulfone concentrations in rat plasma dedicated to pharmacokinetic studies. To evaluate the effectiveness of this method, fipronil was orally administered to rats every day over 13 days at a dose of 3 mg/kg b.w. As shown in Fig. 4, fipronil and fipronil sulfone concentrations ranged from 10.7 ng/mL (±8.6 ng/mL) to 231.0 ng/mL (±67.3 ng/mL) and from 42.7 ng/mL (±11.6 ng/mL) to 2817.6 ng/mL (±535.4 ng/mL), respectively, illustrating the need for a reliable method allowing a wide range of concentration measurements. The mean ratio of the fipronil sulfone concentration to fipronil was 0.4 after the first oral administration and 80 after the last administration. This method enabled fipronil and fipronil sulfone plasma exposure to be monitored without dilution for high concentration samples while quantifying low concentrations in the same run.

4. Conclusion

The present analytical method describes the quantification of fipronil and fipronil sulfone in rat plasma by LC/UV/MS/MS. The volume of the plasma sample for extraction has been reduced to 75 μL while ensuring a limit of quantification within the ng/mL range. This method has been validated according the criteria of the FDA guidelines recommended for pharmacokinetic studies in 2001. The particular advantage of this method relies on its ability to quantify both fipronil and fipronil sulfone over a wide range of concentrations (2.5–2500 ng/mL) in a single run. This represents a real benefit in terms of size of blood sample and time devoted to assay.

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